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Mosquito appetite for blood is stimulated by *Plasmodium chabaudi* infections in themselves and their vertebrate hosts

Heather M Ferguson*[1,2,3] and Andrew F Read[1]

Address: [1]Institute of Cell, Animal and Population Biology Ashworth Laboratories, King's Buildings University of Edinburgh, Edinburgh, U.K., EH9 3JP, [2]Ifakara Health Research and Development Centre P. O. Box 53, Off Mlabani Passage Ifakara, Tanzania and [3]Laboratory of Entomology, University of Wageningen P. O. Box 8031, 6700 EH Wageningen The Netherlands

Email: Heather M Ferguson* - Heather.Ferguson@wur.nl; Andrew F Read - a.read@ed.ac.uk

* Corresponding author

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Abstract

Background: Arthropod vectors of disease may encounter more than one infected host during the course of their lifetime. The consequences of super-infection to parasite development are rarely investigated, but may have substantial epidemiological and evolutionary consequences.

Methods: Using a rodent malaria model system, behavioural avoidance of super-infection was tested by examining whether already-infected *Anopheles stephensi* mosquitoes were less responsive to new vertebrate hosts if they were infected. Additionally, a second dose of parasites was given to malaria-infected mosquitoes on a biologically realistic time scale to test whether it impeded the development of a first infection.

Results: No effect of a second infected blood meal on either the prevalence or parasite burden arising from a first was found. Furthermore, it was found that not only were infected mosquitoes more likely to take a second blood meal than their uninfected counterparts, they were disproportionately drawn to infected hosts.

Conclusions: The alterations in mosquito feeding propensity reported here would occur if parasites have been selected to make infected vertebrate hosts more attractive to mosquitoes, and infected mosquitoes are more likely to seek out new blood meals. Although such a strategy might increase the risk of super-infection, this study suggests the cost to parasite development is not high and as such would be unlikely to outweigh the potential benefits of increasing the contact rate between the parasite's two obligate hosts.

Background

Many arthropod disease vectors have multiple opportunities to become infected with the same pathogen species during their lifetime (super-infection). The impact of super-infection within vectors to parasite transmission is largely unknown, and may have substantial impacts on epidemiology. For example, in the laboratory, pathogen transmission can be enhanced when different parasite species co-occur in the same individual vector, a phenomenon that has been observed in some [1-4] but not all mosquito species that have been tested [1,4].

The aim of this study was to investigate the potential epidemiological consequences of super-infection of mosquitoes by malaria parasites. Super-infection of vectors by successive parasite infections has been examined in a

variety of infectious diseases [5-7], but to knowledge, the frequency and outcome of malaria super-infection has never been investigated. Malaria parasites are a relevant model for studies of vector super-infection because their biology dictates a substantial risk of super-infection in the wild. First, female *Anopheles* mosquitoes try to blood feed at least once every two to four days [8], so that mosquitoes can receive two or more separate infections during their life. In the wild, approximately 20 % of *An. gambiae* mosquitoes live through two feeding cycles, with 6% living four or more [9,10]. Second, a substantial proportion of some *Anopheles* spp. return to the same house on different feeding cycles [e. g. [11]], so that mosquitoes that encounter infected blood are likely to do so again, especially if infected hosts are more attractive to mosquitoes as indicated in some animal models [12,13].

A model system consisting of the mosquito *vector Anopheles stephensi* and the rodent malaria parasite *Plasmodium chabaudi* was used to test whether mosquito feeding behaviour could facilitate or diminish the probability of super-infection. Mosquitoes were fed on infected or uninfected blood and then, four days later, offered a second blood meal of either infected or uninfected blood. The propensity to take a second blood meal was observed, as was whether the development of malaria parasites from the first feed was impeded by the introduction of a second infectious meal on a time-scale mimicking natural blood-feeding behaviour. These experiments provide a first insight into a previously unstudied phenomenon that could affect malaria epidemiology.

Methods

Anopheles stephensi were reared as described elsewhere [14], under standard insectary conditions of 70% RH (± 10%) and 27°C (± 3°C). At this temperature, the sporogonic cycle of *P. chabaudi* takes approximately 12–16 days [15]. *Plasmodium chabaudi* was first isolated from its natural host, the thicket rat *Thamnomys rutilans*, in the Central African Republic in 1969–1970. Since then, this parasite has been stored in liquid nitrogen at the University of Edinburgh. In this experiment one clone of *P. chabaudi* was used, known as CR, which was isolated from the original samples [16]. Three inbred female mice (C57BL/6J, Harlan England) of similar age and weight were infected with a dose of 10^5 CR parasites, with three others being left uninfected to act as controls. The control group were given sham injections that contained only the inoculation medium of calf serum and ringers solution. Two days later, a second group of six mice were infected in the same way, with six being sham-injected to act as controls.

Parasitaemia and gametocytaemia were estimated from thin smears taken from tail blood that were examined under a compound microscope (100 ×) as the proportion of red blood cells (RBC) in a random sampling of 300 that were infected with asexual parasites, and the proportion of gametocyte-infected red cells in a random sample of 5000–10,000 RBC respectively. A few hours before mosquitoes were fed on mice, RBC densities were estimated from a 2 μl sample of tail blood by flow cytometry (Coulter Electronics, Luton, England). Asexual and gametocyte densities were estimated as the product of RBC density and parasitemia or gametocytaemia respectively.

Mosquito feeds

Groups of 250 pupae were randomly selected from the rearing trays 10–13 days post-egg hatching and placed in one of six emergence cages (16 × 16 × 16 cm), giving rise to 160–240 adults that were fed *ad libitum* on a 10% glucose solution supplemented with 0.05% PABA. Mosquito feeds on the first group of infected and control mice (n = 6) took place 16 days after infection, when gametocytes were detectable in all infected mice. Mosquitoes were 4–5 days old at the time of first blood feeding. To increase their appetite, mosquitoes were deprived of glucose for 24 hours before the blood feed. To feed, one anaesthetised mouse was placed on top of each cage and mosquitoes were allowed to bite for 20 minutes.

Immediately after the first blood feeding trial, mosquitoes that had not fed were removed. Ten fully engorged mosquitoes were transferred individually into 30 ml plastic tubes (9 × 2.5 cm) covered with mesh, with the rest being left in the original six cages. A 10% glucose solution (plus 0.05% PABA) was provided *ad libitum* to mosquitoes held both in cages and in tubes (in cages, glucose was supplied by filter paper wicks, and in tubes by cotton pads soaked in solution that were placed on the top netting).

After 3 days, mosquitoes in tubes were returned to the treatment cages from which they were taken. Hematin within these holding tubes was quantified using a standard photometric assay (as described in [17]) to provide an estimate of blood meal size. On the evening of this same day, a water-filled petri dish was placed in each cage to allow blood-fed females to lay their eggs. These dishes were removed the next morning.

A second blood feed was offered to mosquitoes four days after the first. Prior to the feed, the original six mosquito populations were each split into two new cages, with one receiving an infected host and the other an uninfected host. Twelve mice were prepared for use in this feed (inoculated 18 days before), six infected and six uninfected. However, due to a combination of factors including mouse death prior to and during the feed day, and the failure of gametocytes to develop in some mice, only eight mice (four control and four infected) could be used to feed mosquitoes in each of the 12 cages (with each mouse

being used to feed at least two cages). Successive feeding of mosquitoes in different cages from the same mouse was possible because once anaesthetized, mice remain unconscious for over an hour (sufficient for 2 × 20 minute feeding trials), and the relatively small number of mosquitoes per cage (average = 30) ensured that the blood loss per feed was minimal. Any mouse that died during the course of the second blood feed was immediately replaced by a live one from the same infection treatment.

After the second blood feed, all mosquitoes (fed or not) were moved into individual 30 ml tubes for hematin collection, as described above. After 3–4 days (7–8 days after first blood meal), mosquitoes were killed using chloroform, and potentially infected mosquitoes dissected under a microscope (10 ×) in a drop of phosphate-buffered saline (PBS) and inspected for oocysts. From prior experience it is known that at this magnfication, oocysts that have been growing for 7–8 days are easily distinguishable, whereas those that are only 3–4 days old are not. Thus it was certain that all detected oocysts had arisen from the first infectious blood meal.

One wing was removed from all mosquitoes and measured using an ocular micrometer to provide a measure of body size. Haematin that accumulated in the bottom of each tube was quantified as described above. Mosquitoes from tubes where the haematin absorbance was <0.1 nm, the level found in the lithium carbonate control, were classified as a non-feeders. All experiments described above were conducted in accordance with the British Home Office regulations for animal experimentation.

Statistical analysis

The two main questions were (1) does the infection status of the first blood meal (infected or uninfected) influence the propensity of mosquitoes to feed when presented with a second host, and (2) does exposure to parasites in a second blood meal alter the infectivity of parasites from the first? To answer these questions, a series of different statistical models was applied to the data (as described in Table 1[18]). Explanatory variables included the status of the first and second feed (infected or uninfected), with 'mouse'-specific effects (nested within infection treatment) also being fitted to control for additional sources of variation. When the response variable had a binary outcome (presence of parasites, probability of taking a second blood meal), logistic regression was applied to the data using the PROC GENMOD subroutine in SAS, incorporating binomial errors. When the response variable was continuous (number of oocysts, eggs, and size of blood meal), mixed models ANOVA was applied where the main effect of host infection status was treated as a fixed effect, and individual mice as random factors. Differences between mice within treatments were often significant, but as they are of no interest in their own right, they were controlled for by leaving 'mouse-within-treatment' terms in models when they were significant.

Table 1: Description of statistical models applied to analyse the influence of first and second blood meal type (*P. chabaudi* infected or uninfected) on the feeding behaviour and infection susceptibility of *An. stephensi* mosquitoes. In 'Group Analysed', details of the subset of mosquitoes included in a particular analysis are given, with 'first feed' indicating the type of blood meal they were first given (I – only mosquitoes first fed infected blood, I + U – mosquitoes whose first feed was infected or uninfected). In analyses of mosquitoes of whose first feed was infected (models 2,3,5–8), the 'oocyst present' column indicates whether all mosquitoes were included (-), or just those that developed oocysts (Y). The 'took a second feed' column indicates whether analysis was performed on all mosquitoes that had a first blood meal (-), or just those who took a first and second blood meal (Y). N gives the number of mosquitoes included in each analysis. 'Maximal Model' gives the complete set of factors in addition to wing size that were included as explanatory variables for each response variable, with terms in brackets indicating nested variables. Explanatory variables are: FEED1 – status of first blood meal (I or U), FEED2 – status of second blood meal (I or U), with MOUSE1 and MOUSE2 representing the particular mouse within each treatment group that mosquitoes fed on in the first and second feeding trial, respectively. The superscript[a] denotes variables that were fit as random effects, all others being treated as fixed effects.

Parameter of Interest	Response Variable	Group Analyzed			N	Model No.	Maximal model (not listing wing size)	SAS subroutine
		First feed	Oocysts present	Took a 2nd feed				
Blood Feeding	Had a 2nd blood feed	I + U	-	-	352	1	F1 + F2(F1) + M1(F1) + M2(F2)	PC
		I	-	-	75	2	Oocyst presence + F2 + M2(F2)	PG
		I	Y	-	45	3	Oocyst number + F2 + M2(F2)	PG
	Log (size of 2nd blood meal)	I+U	.	Y	167	4	F1+ F2(F1) + M1(F1)[a] + M2(F2)[a]	PM
		I	-	Y	51	5	Oocyst presence + F2 + M1(F2)[a] + M2(F2)[a]	PM
		I	Y	Y	34	6	Oocyst number + F2 + M1(F2)[a] + M2(F2)[a]	PM
Infection Rate	Oocyst presence	I	-	Y	54	7	F2 + M1	PG
	Log(oocyst no.)	I	Y	Y	34	8	F2 + M1 [a]	PM

Maximal statistical models included the explanatory variables listed in Table 1 and wing size. Mosquito body size (as indexed by wing size) is an important determinant of blood meal size, blood feeding tendency and possibly *Plasmodium* infection rate [19,20], all of which were response variables in this study. Wing size was included as an explanatory variable in our analyses in order to control for this extra source of variability in order to clarify main treatment effects.

Non-significant terms were sequentially dropped to yield the minimum statistically significant description of the data. Prior to analysis, data on the number of oocysts per mosquito and the size of their second blood meal were log transformed to increase their fit to the normal distribution (as assessed by the Kolmogorov-Smirnov normality test [21]).

Results

Feeding behaviour following infection

A total of 523 mosquitoes were offered an initial blood meal, of which 83% fed during the 20-minute exposure period. Of mosquitoes that took a first blood meal, approximately 47% fed again when offered a second blood meal four days later. Of the 193 mosquitoes whose first blood meal was infected, 55% took a second blood meal when offered 4 days later.

The propensity of mosquitoes to take a second blood meal was strongly associated with the infection status of their first blood meal (Figure 1; $\chi_1^2 = 22.14$, $p < 0.01$). Mosquitoes whose first meal was infected were almost one and a half times more likely to take a second meal than those whose first blood meal was uninfected (proportion taking a second feed: 0.39 and 0.56 for uninfected and infected mosquitoes respectively). Additionally, mosquitoes were more likely to take a second blood meal if the host they were presented with was infected (Figure 1; Feed2(Feed1): $\chi_1^2 = 7.95$, $p < 0.01$). Among the 75 mosquitoes first fed infected blood and surviving until dissection on days seven or eight, those that went on to develop oocysts were somewhat more likely to take a second blood feed than those that did not (Model 2: $\chi_1^2 = 3.06$, $p = 0.08$). There was no evidence that feeding tendency increased with the number of oocysts within a mosquito (Model 3, $\chi_1^2 = 0.41$, $p = 0.52$, range = 1–249).

Of mosquitoes that blood-fed twice, the size of the second meal was not influenced by the parasite status of the first (Model 4, Feed2(Feed1): $F_{2,6} = 1.55$, $p = 0.28$). Amongst mosquitoes whose first meal was infected, neither the presence (Model 5, $F_{1,31} = 1.68$, $p = 0.20$) nor number of oocysts (Model 6, $F_{1,26} = 0.36$, $p = 0.55$) influenced the size of the second blood meal. Thus, exposure to parasitized blood in the first or second feed influenced the propensity to take a blood meal, but not the size of the meal.

Re-exposure to parasites and infection

Sixty-three percent of mosquitoes whose first meal was infected went on to develop oocysts, with a mean oocyst intensity of 28.5 (s.e. ± 6.1). Amongst this group, there was no evidence that exposure to parasites in a second feed reduced the development of parasites acquired during the first feed. The presence of gametocytes in the second blood meal had no effect on the oocyst rate arising from the first (Figure 2, $\chi_1^2 = 1.31$, $p = 0.25$). Amongst mosquitoes with oocysts after their first infectious meal, the parasite status of their second blood meal did not influence the number of oocysts they developed ($F_{1,29} = 0.05$, $p = 0.81$). The best predictor of infection rate following the first blood meal was the identity of the first infectious mouse ($\chi_1^2 = 14.56$, $p < 0.01$), with prevalence being highest in mosquitoes fed on mice with the most gametocytes on the day of blood feeding.

Discussion

This study has demonstrated that the presence of malaria parasites in either of their two obligate hosts increases the probability of contact with the other. *Plasmodium*-infected mosquitoes were not only more willing to take a second blood meal than their uninfected counterparts, but were almost 1.3 times more likely to initiate feeding if that host was also infected (Figure 1). This phenomenon would increase the risk of super-infection in mosquitoes beyond that expected under the assumption of random contact between hosts and vectors.

Super-infection is probably detrimental to mosquitoes, given that even infections acquired just once reduce mosquito fecundity [22-27] and in some cases, survival [14,28-31]. Why then would vectors, or indeed the parasites that depend upon their survival for transmission, have or induce a feeding strategy that increases the risk of multiple infection? Perhaps the most parsimonious explanation is that natural selection on malaria parasites has acted to make infected vertebrate hosts more attractive to mosquitoes, and infected mosquitoes more willing to blood feed. Both traits would increase contact rate between *Plasmodium's* two obligate hosts, probably the most important limiting factor to malaria epidemiology. Although such a strategy would engender an increased risk of super-infection, it would none-the-less thrive if the fitness costs of co-existence were minimal.

Quite how *Plasmodium* infection makes an anaesthetised mouse more attractive is unclear, but there have been numerous reports that other vector-borne parasites increase the attractiveness of their mammalian hosts [32-37]. In malaria, increased feeding on infected hosts has

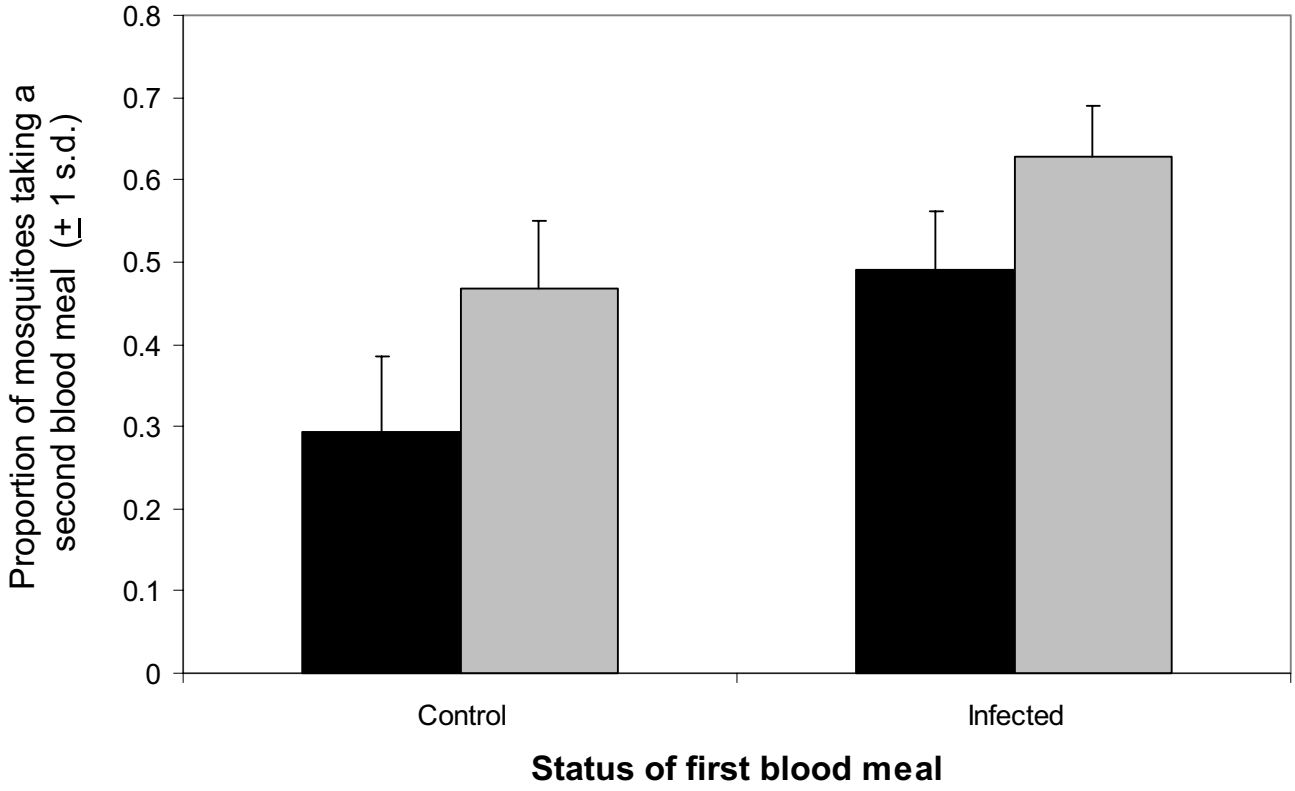

Figure 1
Proportion of mosquitoes that took a second blood meal as a function of the first blood meal they imbibed. Solid black bars indicate that the second blood meal was taken from an uninfected host, and grey bars that the second host was infected with *P. chabaudi* gametocytes. Error bars represent one standard deviation (calculated for the binomial distribution).

been reported in some [12,38,39], but not all cases [27,40]. Other studies have shown that malaria-infected mosquitoes are more persistent in seeking out blood meals, and bite more often than their uninfected counterparts [41-43]. Some of these studies reported that malaria-infected mosquitoes increased their biting frequency only when infected with the transmissible sporozoite stage of the parasite, and not when oocysts are present [41,44,45]. It has been argued that this apparent stage-specificity is a product of natural selection acting to maximize contact rate with vertebrate hosts only when the parasite is actually capable of being transmitted [44,45]. It is unclear why oocysts apparently enhance feeding propensity in this study (Figure 1) but depress it in others. The energetic demands put on mosquitoes by growing oocysts may, in our system, lead to increased desire for nutrient intake. We have previously observed increased sugar-feeding by oocyst-infected *An. stephensi* [46], an observation that now appears to extend also to blood-feeding. However, it seems unlikely that the enhanced blood-feeding reported here can be explained solely as a function of parasite energetic demand, because feeding propensity was unrelated to oocyst burden. Further investigation of mosquito blood-feeding behaviour throughout all stages of parasite development, and under resource rich and poor conditions, may help determine the conditions under which oocysts enhance or suppress feeding tendency.

Given that mosquito feeding behaviour appears to enhance the risk of super-infection, the question remains as to what, if any, fitness costs it elicits either on the part of the parasite or the vector. The feeding preferences reported here would not increase the risk of super-infection were infected hosts more likely to exhibit anti-vector behaviour and infected mosquitoes more likely to succumb to it, a possibility we did not test here. Additionally, although no evidence that the early development of malaria parasites was impeded by re-exposure to parasites was found here (Figure 2), the possibility of suppression (or indeed enhancement) during the later part of the spo-

Figure 2
Proportion of mosquitoes that were initially fed infected blood that developed oocysts as a function of the parasite status of a second blood meal. The second blood meal was given to mosquitoes four days after the first. The data represent the average infection rate across 3 trials (51 mosquitoes), with bars indicating one standard error.

rogonic cycle cannot be ruled out. Indeed, we only tested for a negative development effect of super-infection over a limited period of the sporogonic cycle (oocyst development between day 4 and 7). Had we tracked infection success through to the sporozoite stage, we may have detected a developmental effect of superinfection. Malaria parasite growth in mosquitoes can be hindered by subsequent infection with filarial worms [47], and this may have happened here at a later stage of infection. Additionally, this study was restricted to examining the development of a first *Plasmodium* infection in the presence of a second (not the development of a second infection in the presence of the first). Thus we have only described the fitness consequences to one party in the super-infection. Although the successful development of a first infection may not be hindered by a second, it is possible that the second cannot develop in the presence of the first. Quantitative PCR protocols to track the relative frequencies of primary and superinfecting clones in sporozoite populations are currently being developed. Such tools will allow testing of whether any of the additional potential costs of super-infection proposed here exist, and whether they could impose sufficient selection to counteract the feeding-behaviour enhancement we report in this study.

Conclusions

This study demonstrated that malaria-infected mosquitoes are not only more likely to seek out a second blood meal than their uninfected counterparts, but also that on second feeding, infected mosquitoes are disproportionately drawn to infected vertebrate hosts. This phenomenon may be the product of natural selection acting on parasites to enhance their transmission risk by increasing the contact rate between their two obligate hosts. One potential drawback of this strategy is that it would engender an increased risk of super-infection in mosquitoes, which could hamper parasite and/or vector fitness. However, we found no evidence that parasite

development in mosquitoes was impeded by re-exposure to *Plasmodium* on a second meal. Future studies that examine the impact of *Plasmodium* super-infection on mosquito longevity will be of great use to resolving whether the potential transmission benefits of the feeding behaviour shifts described here could be counteracted by increases in vector mortality.

Authors' contributions

HF designed, conducted and analysed the experiments described here, and wrote the manuscript. AR assisted in drafting the final manuscript, planning the experiments, and obtained the Wellcome Trust grant with which this work was funded.

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